

(51) Int. Cl.⁴ ID. No.
G 01 N 33/50
C 12 N 15/00
C 12 Q 1/58

Internal Filing. No.
P - 8305 - 2G
7115 - 4B
8213 - 4B

(43) Publication Date: December 2nd, 1985

Examination Request: Not Filed
No. of Inventions: 1
(Total Pages: 5)

(54) Title of the Invention: METHOD OF DETERMINING NUCLEIC ACID BASE SEQUENCES

(21) Application No.: S59 - 96454

(22) Application Filed: May 16, 1984

(72) Inventor: Yoshinori HARADA
c/o Hitachi Central Research Laboratory
1 - 280 Banchi, Higashi Koigakubo, Kokubunji City

(72) Inventor: Yasushi SHIMADA
c/o Hitachi Central Research Laboratory
1 - 280 Banchi, Higashi Koigakubo, Kokubunji City

(72) Inventor: Hideki JIMBARA
c/o Hitachi Central Research Laboratory
1 - 280 Banchi, Higashi Koigakubo, Kokubunji City

(72) Inventor: Keiichi NAGAI
c/o Hitachi Central Research Laboratory
1 - 280 Banchi, Higashi Koigakubo, Kokubunji City

(71) Applicant: Hitachi Ltd.
4 - 6 Surugadai, Kanda, Chiyoda-ku, Tokyo

(74) Patent Representative: Akio TAKAHASHI, Patent Attorney (and one other)
(Continued on last page)

Specification

Title of the Invention: METHOD OF DETERMINING NUCLEIC ACID BASE SEQUENCES

Range of Patent Claims

1. A method of sequencing ribonucleic acid sequences with the following features. Deoxyribonucleic acid (DNA) samples are divided into fourths and each fourth is joined to fluorescent pigments having differing excitation wavelengths. Next, base-specific chemical reactions are carried out and analyses performed using electrophoresis methods based on individual phoretic paths.
2. The method of determining ribonucleic acid sequences described in Claim 1 of the range of patent claims with the following characteristics. The aforementioned fluorescent pigments have different light-emitting spectra.
3. The method of determining ribonucleic acid sequences described in Claim 1 or Claim 2 with the following characteristics. The aforementioned process of bonding with the fluorescent pigments is carried out after the previously described chemical reaction.
4. The method of determining ribonucleic acid sequences described in Claim 1 through Claim 3 with the following characteristics. The analyses are performed using liquid chromatography.

Detailed Description of the Invention

The Invention's Fields of Use

This invention involves methods of determining deoxyribonucleic acid (DNA) base sequences. In particular, it pertains to those methods of determining deoxyribonucleic acid base sequences that are capable of separation, the detection of favorable DNA and can be made more precise and faster.

Background of the Invention

A mixture of DNA fragments is separated using electrophoresis methods, organized in order of molecular weight and detected. Traditionally, when doing this, a radioactive isotope like ^{32}P or ^{35}S or a biotin fluorescent pigment is used to mark the end of the DNA molecules before electrophoresis or, the sample is dyed after electrophoresis using silver (Proteins/Ribonucleic Acids/Enzymes), ethidium bromide, acrylidine orange, proflavine or other fluorescent pigment (Proteins/Ribonucleic Acids/Enzymes Special Issue: Principles of Fluorescent Measurements and Applications with Biosystems, pp. 206 – 231) in order to detect the electrophoretic separation bands of the DNA fragments. However, the dyeing method did not allow base type identification in any of these cases.

For this reason, according to traditional methods of determining DNA base sequences (Methods in Enzymology, 65, pp. 449 – 580), even after base-specific DNA strand cutting reactions were carried out on at least 4 types of DNA fragments or a reaction to halt the generation of base-specific complementary strands was carried out, it was necessary to separate each of the phoretic paths by reaction type for the electrophoretic separation of the

ribonucleic acid fragment.

Figure 1(a) shows a model diagram of the electrophoretic separation of substances generated by four types of base-specific reactions A, C, G, and T, using conventional methods. In this example, the phoretic bands were detected using marking methods and proprietary detection methods and the DNA base sequences were determined using the order of the degree of movement and which phoretic path the detection took place in. In this example, it is TGCAACGATTCGGCATGACG. However, with conventional electrophoresis methods, distortions of the sort shown in Figure 1(b) in the electrophoretic separation image were a frequent occurrence, caused by unsuitable phoresis conditions. It was difficult to determine the order of the degree of movement, that is, the base sequence.

Purpose of this Invention

The purpose of this invention is to provide a method that makes it possible to simplify and improve the precision and speed of determining DNA base sequences by mixing DNA fragments that have undergone many types of base-specific reactions and, separating them into DNA fragments that have various degrees of movement using either an electrophoresis method that uses individual phoresis paths or a liquid chromatography method.

Summary of the Invention

In order to eliminate the lack of precision surrounding the relative positions of the phoresis bands of the DNA fragments caused by the independent separation of base-specific resultants during each reaction, this invention employs the following means. The intent is to simplify while improving the precision and speed of the DNA base sequence determination.

(1) Marks are made using 4 types of base-specific DNA strand cutting reactions or else the nucleic acid is marked using 2 to 4 types of fluorescent pigment having different qualities so that the 4 types of resultants can be distinguished before the complementary strand-generation reaction process. (In order to distinguish 4 types of substances, at least 2 types of independent markers will be necessary.)

(2) Additionally, marks are made using fluorescent pigments having 4 types of different characteristics so that the 4 types of reactions can be distinguished after the 4 types of base-specific DNA strand cutting reactions or the complementary strand generation reaction.

(3) The base-specific resultants described in (1) and (2) above are separated by molecular weight using either an electrophoresis method or a high-speed liquid chromatography method.

(4) When using pigments having differing excitation wavelengths as the fluorescent pigment markers described in (1) and (2) above, the light source having the corresponding number is to be used.

(5) When using different light-emitting spectra as the fluorescent pigment markers described in (1) and (2) above, a spectrometer that can split the light-emitting spectra or a filter and a light collector are to be used.

Embodiments of the Invention

An embodiment of this invention will be described below with reference to Figure 2. Figure 2(a) shows the aligned images (mass spectrum) of DNA fragments for each base-specific reaction along a single phoresis path created using the 4 types of fluorescent pigment (such as etheno-nucleotides). That is, the DNA fragments produced using DNA strand cutting reactions or CNA complementary strand generation reactions in this embodiment are marked using excitation wavelengths or 4 types of fluorescent pigments that have different maximum light-emission wavelengths. For this reason, the use of a detection method that corresponds to the method of marking (either excitation at different wavelengths or the splitting of the emitted light into its spectrum) will allow the mass spectra of the four types of resultants to be analyzed even if a single phoresis path is used to separate the mix. Phoresis is carried out under the same conditions using a single phoresis path, so even if there is the type of phoretic separation image distortion shown in Figure 1(b), the relative positions between the phoretic bands will be maintained, so a highly accurate determination of the DNA base sequences can be made.

Figure 2(b) shows the mass spectra of DNA fragments made for each base-specific reaction using 2 types of fluorescent pigment. According to this embodiment, of the DNA strands generated using a DNA strand cutting reaction or a DNA complementary generation reaction, the A and C resultants are marked with the pigment 1 and the A and G resultants are marked with the pigment 2. This means that the 4 types of resultants are mixed. If, after undergoing phoretic separation in the same phoretic path, they are detected using a detection method that corresponds to the marking method, then 4 types of detection results will be obtained. That is, in addition to the phoretic bands dyed using the pigments 1 and 2 (these are abbreviated to (+, +)) are the phoretic bands (+, -), (-, +) and (-, -). In this example, these correspond to A, C, G and T, so their mass spectra can be interpreted. And, as shown in Figure 1(b), even in this embodiment, when the electrophoresis is carried out using the same phoretic path as the mixture of nucleic acid fragments and under the same conditions, there are no difficulties interpreting the

mass spectra even if you end up with distortion in the phoretic separation images. For this reason, DNA sequence determinations can be made with a high degree of precision.

As above, it is clear that it would be possible to identify the 4 types of resultants even when if 3 types of fluorescent pigment were used in the second embodiment.

Figure 3 shows 4 types of devices based on combinations of 2 types of fluorescent markers ((I) Using excitation wavelength differences and (II) Using fluorescent spectral differences) as well as 2 types of mass spectrum-generating methods ((I) Electrophoresis method and (II) Liquid chromatography). In Figure 3(a), in order to distinguish the 4 types of base-specific resultants, they were marked 12 individually with fluorescent pigments having differing excitation wavelengths, mixed 1 and after they had been separated on a single phoresis path 2, the fluorescent light was detected 7 as the phoretic separation band 3 was sequentially excited and discharged at difference wavelengths ($\lambda_1 \sim \lambda_4$). If the time relationship between the excitation signal and the emitted light is analyzed, the base-specific reaction that generated the DNA fragments can be determined without running a spectral analysis on the fluorescent light. For this reason, this method identifies the phoretic bands that appear sequentially making it possible to determine the DNA base sequences. Figure 3(b) is an embodiment showing the use of liquid chromatography instead of electrophoresis as a method for creating mass spectra of the DNA fragments. The results are the same as in (a). In Figure 3(c), in order to identify the 4 types of base-specific resultants, they are each individually marked 13 with 4 types of fluorescent pigment having different emitted spectra in response to the excitation of a single wavelength or continuous light. They are then mixed 1 and after they are separated using a single phoretic path 2, the pigment is excited at a specific wavelength, spectral analyses 8 are run on the phoretic separation bands and they are detected 7. Filters could be used instead of spectral analysis. In this system, the exciter light source module is simpler than the 2 embodiments described above. Figure 2(d) shows an embodiment where liquid chromatography was used for the mass spectra of the DNA fragments. The results are the same as in (c).

In the embodiments described above, the DNA fragments in all cases had already been marked using fluorescent pigment prior to the electrophoretic separation. We did not touch on the subject of the time relationship between the marking reaction process that should be completed before the separation, the base-specific reaction process or and the qualities that the fluorescent pigment should have. For this reason, we will discuss these relationships below. First, when the marking reaction is to be carried out before the base-specific reaction, it would be possible to apply this invention in every instance. However, the fluorescent pigment in such instances would have to form a covalent bond with the DNA fragment and would have to be able to tolerate a base-specific reaction. Etheno-adenosine and other etheno bases have this quality. Conversely, when running marking reactions after the base-specific reactions, there could be times that this invention could not be used, depending on the type of base-specific reaction. That is when using a complementary strand generation reaction (Methods in Enzymology, 65, pp. 560 – 580) as the base-specific reaction, the DNA fragments generated after the reaction would all be carrying significant data for determining the DNA base sequences, so these could be marked using fluorescent pigments and mass spectra created. In this case, the only requirement of the pigment would be that it form a base-specific bond with the DNA. There would be no need to form a covalent bond, so the conditions would be less stringent than those described above. However, when using the base-specific DNA strand cutting reaction (Methods in Enzymology, 65, pp. 499 – 560), in the DNA fragments that are generated, fewer than 1/4 of the total will carry data significant to the determination of the DNA base sequences. It is not possible to mark just this portion selectively, so this invention could not be applied in this case.

Effect of the Invention

This invention makes it possible to use molecular weight separation of DNA fragments to make highly precise analyses of spectra by splitting a number of resultants simultaneously and under the same conditions when making mass spectra. As a result, it is possible to automate the determination of DNA base sequences and make the process highly precise.

Brief Description of Drawings

Figure 1 and Figure 2 are explanatory drawings that show the validity of the first embodiment of this invention. Figure 3 is an explanatory diagram that shows the four embodiments of this invention.

1: Marked DNA Fragment Mixture; 2: Electrophoresis Support; 3: Phoretic Separation Band; 4: High Pressure DC Power Supply; 8: Spectrum Analyzer or Filter; 9: Pump for Liquid

10: Film for Liquid Chromatography; 11: Detector Cell; 12: Dyeing Tank that Uses Fluorescent Pigments with Differing Exciter Wavelengths; 13: Dyeing Tank that Uses Fluorescent Pigments that Have Light-Emitting Spectra with Differing Fluorescent Wavelengths; 14: Phoretic Path

Representative: Akio TAKAHASHI, Patent Attorney [illegible stamp]

Figure 1

(a)

(b)

Figure 2

(a)

(a)

Figure 3

(b)

(c)

(d)

(b)

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(53) Int. Cl. ⁴	ID. No.	Internal Filing. No.
G 01 N 21/76		6637 - 2G
33/52		8305 - 2G

(72) Inventor: Jiro KAMODA
c/o Hitachi Central Research Laboratory
1 - 280 Banchi, Higashi Koigakubo, Kokubunji City